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Metabolic processes

Expression of core-binding factor α1 and osteocalcin in fluoride-treated fibroblasts and osteoblasts

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ABSTRACT

To study the effects and importance of fluoride on FBs in the development of extraperiosteal calcification and the ossification of skeletal fluorosis, the presence of the osteogenic phenotype, which is indicated by the expression of core-binding factor α1 (Cbfa1) and osteocalcin (OCN), in an FB cell line (L929) and in osteoblasts (OBs) exposed to fluoride was determined. Fibroblasts and osteoblasts were exposed to different concentrations of fluoride (0, 0.0001, 0.001, 0.1, 1.0, 10.0 and 20.0 mg/L F^-). By using RT-PCR and ELISA, the mRNA levels of Cbfa1 and OCN were measured at 48 h, and the protein levels of Cbfa1 and OCN were measured at 2, 4, 24, 48 and 72 h. The data demonstrated the following: (1) The Cbfa1 protein level in fluoride-treated fibroblasts clearly increased at 48 h in the groups treated with 0.0001, 0.001, 0.1, 1.0 and 20.0 mg/L F^-. The Cbfa1 protein level of the group treated with 10 mg/L F^- at 72 h was higher than that of the control group. The level of Cbfa1 mRNA in the fibroblasts was much higher at 48 h in the group treated with 10.0 mg/L F^- than in the control group. (2) The OCN protein level in fluoride-treated fibroblasts was significantly higher than that of the control group in the 0.0001, 0.1, 1.0, 10.0 and 20.0 mg/L F^- groups at 2 h, and in the 0.001 and 0.1 F^- groups at 4 h. A slightly higher level of OCN mRNA in fluoride-treated fibroblasts was also found in the 1.0 and 20.0 mg/L F^- groups compared to the control group. (3) The expressions of Cbfa1 and OCN in osteoblasts treated with the same experimental conditions as the fibroblasts were up-regulated by fluoride following the same trend as in the fibroblasts. Our results showed an increase in the expression of Cbfa1 and OCN in fibroblasts and osteoblasts exposed to fluoride and suggested that the osteogenic function of fibroblasts induced by fluoride could play an important role in the development of extraperiosteal ossification during skeletal fluorosis.

Introduction

Extraperiosteal calcification and ossification plays an important role in the development of severely disabled skeletal fluorosis. For studying the mechanism of extraperiosteal calcification and ossification during skeletal fluorosis, attention is focused on the fibroblast (FB), which is the main cell type existing in the extraperiosteal soft tissue, such as tendons and the linking points of ligaments. FBs are considered inducible osteogenic precursor cells (IOPC) because of their osteogenic function under non-physiological conditions [1]. In this study, we proposed that fluoride can stimulate the osteogenic function of FBs by enhancing the expression of osteogenic markers. Although many details remain to be studied, the interaction between fluoride and FBs is strongly analogous to the mechanism of extraperiosteal ossification. Furthermore, it has been shown that core-binding factor α_1 (Cbfa1) and osteocalcin (OCN) are special markers of osteoblast-like cell lines [2,3]. Cbfa1, an essential transcription factor in osteoblast differentiation and bone formation [4–7] plays an essential role in osteogenesis [8–10]. Furthermore, studies have shown that Cbfa1 plays important roles in matrix production and mineralization [11,12]. Osteocalcin, a major noncollagenous matrix protein of bone, dentin, and cementum, is found in tight association with the calcium phosphate mineral phase of these tissues [13]. Consequently, we focused on the expression of Cbfa1 and OCN in FBs treated with fluoride to explain the mechanism of extraperiosteal ossification during skeletal fluorosis with the future goal of developing a cure for this disease.

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Table 1
Primer sequences.

Accession number	Gene name	Forward and reverse primer	Position	Size (bp)
BC068988.1	β-Actin	5′-CATCTCTTGCTCGAAGTCCA-3′ 5′-ATCATGTTTGAGACCTTCAACA-3′	430–733	317
NM_007527.2	Cbfa1	5′-CACGACAACCGCACCATG-3′ 5′-GTCCCATCTGGTACCTCTCCG-3′	234–598	166
BC013447.1	OCN	5′-ATGAGAGCCCTCAGACTCCTC-3′ 5′-CGGGCCGTAGAAGCGCCGATA-3′	1553–1800	296

Materials and methods

Cell culture and fluoride treatment

Fibroblast

The fibroblast cell line L929 was purchased from cell bank (The Chinese Academy of Sci.) and cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal calf serum (FCS, Gibco, USA) at 37 °C in a humidified atmosphere of 5% CO_2/95% air. Before fluoride treatment, the cells were cultured in DMEM containing 5% FCS for 24 h. The MTT results showed that the proliferous activity increased significantly in FBs exposed to certain range of fluoride concentration (0.001 mg F^-/L) at certain time (1 h and 2 h). The proliferous activity decreased with the time of culture and the doses increase of fluoride [14]. Thus, the fibroblasts of the experimental groups were treated with DMEM (5% FCS) containing different concentrations of fluoride (0.0001, 0.001, 0.1, 1.0, 10.0 or 20.0 mg/L) for 2, 4, 24, 48 and 72 h. Cells in the control group were cultured with DMEM (5% FCS) for the same time points.

Osteoblast

Primary calvarial osteoblasts were prepared from Kunming mice. Neonatal mouse calvaria were dissected free from adherent soft tissue, cut into pieces and sequentially digested with 0.25% trypsin for 30 min at 37 °C. Then, the cells were digested twice (1 h at 37 °C each time) with 1 mg/mL collagenase (Sigma, St. Louis, MO, USA). After this digest, the cells were washed in phosphate buffered saline (PBS) and centrifuged at 1000 rpm for 5 min, and the pelleted cells were resuspended in an α-modification of Eagle's medium (α-MEM, Gibco, Grand Island, NY, USA) supplemented with 10% calf serum, 100 units/mL penicillin and 100 units/mL streptomycin. Then the cells were seeded into 25 mL flasks (Falcon; BD BioSciences, USA) and grown at 37 °C in a humidified atmosphere of 5% CO_2/95% air. The cells were identified as osteoblasts by alkaline phosphatase staining. OBs were divided into 7 groups and treated in the manner as the fibroblasts (see Section "Fibroblast")

Enzyme-linked immuno sorbent assay (ELISA)

To quantify the Cbfa1 and OCN protein levels in the cell culture supernatants, commercially available ELISA kits were used according to the manufacturer's instructions (Promega, Madison, WI, USA). All measurements were performed in duplicate. Constitutive protein secretion was analyzed following 2, 4, 24, 48 and 72 h of cell culture with different concentrations of fluoride.

Immunohistochemistry (IHC)

Immunohistochemistry was performed on fibroblasts seeded on slides. The fibroblasts were fixed in 10% formaldehyde for 30 min and washed thoroughly with PBS. To inactivate the endogenous peroxidase, the fibroblasts were permeabilized in a solution containing 30% H_2O_2 and methanol (1:50) for 30 min. This step was followed by an incubation with the primary antibody against Cbfa1/Runx2 (Santa Cruz Biotechnology, Inc) overnight at 4 °C, and then the slides were washed three times in PBS. The results were visualized using diamino benzidine (DAB) to generate a brown precipitate, and, finally, the cells were counterstained in hematoxylin for 10 min, dehydrated, made transparent and mounted.

Reverse transcription polymerase chain reaction (RT-PCR)

Before fluoride treatment, the cells were cultured in 5% FCS for 24 h. The fibroblasts were treated with DMEM (5% calf serum) containing fluoride at a concentration of 0, 0.0001, 0.001, 0.1, 1.0, 10.0 or 20.0 mg/L for 2, 4, 24, 48 and 72 h. Total RNA was extracted using TRIzol reagent (Invitrogen Inc., USA) and quantified using a spectrophotometer. Reverse transcription of 5 μg of cultured cell total RNA was performed for 50 min at 42 °C and then 15 min at 70 °C using the Super Scrip First-Strand Synthesis System for RT-PCR (Invitrogen), which contains RT buffer, Oligo 12–18, 5× First-Strand Solution, 10 mM dNTP, 0.1 M DTT, Super Script II (RT-enzyme), and Rnase H (RNase inhibitor). PCR using primers for unique sequences in each cDNA was carried out in a 25 μL reaction mixture that included 2.5 μL Tag buffer, 0.5 μL Tag, 2 μL dNTP, 0.5 μL each primer, 3 μL template and 16 μL DEPC water. For PCR, we used the primers as Table 1. The thermal cycling conditions were as follows: (1) initial denaturation at 94 °C for 2 min, (2) cycling for the cDNA-specific annealing temperature for 30 s and 72 °C for 2 min, (3) final extension at 72 °C for 5 min. The expected size of the cDNA and the primers used are shown in Table 1. An equal volume from each PCR reaction was analyzed by 2% polyacrylamide gel electrophoresis, and the gel was stained with ethidium bromide. The specific bands were quantitatively analyzed by scanning volume density using Tanon GIS-1000 densitometer and GISID software (Tanon Inc., China) and normalized to β-actin levels as an internal control. All the following assays were performed in triplicate.

Statistical analysis

The SPSS program (version 13.0) for windows (SPSS, Chicago, IL, USA) was used for the statistical analyses. Values are given as the means ± standard deviations (SD). A one-way analysis of variance (ANOVA) was used to evaluate the significant differences among group means at each time point. When ANOVA indicated a significant difference between the means, the differences were evaluated using the Fisher protected least significant difference (PLSD). *P* values less than 0.05 were considered to be significant. The graphs of the experimental results were generated with Graphpad Prism 5 software.

Results

The content of Cbfa1 protein in fibroblasts and osteoblasts treated with fluoride

Our IHC results showed that the Cbfa1 protein was located in the cytoplasm in fibroblasts, and positive staining of fibroblasts was only observed in the group treated with 0.0001 mg/L

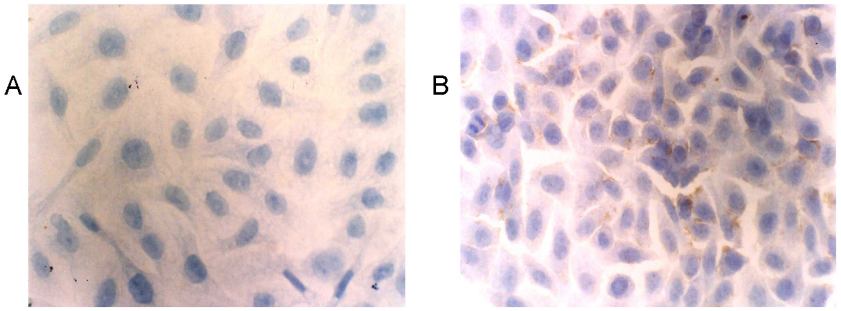

Fig. 1. Effects of fluoride on the expression of Cbfa1 protein in fibroblast (DAB 400×) (A: control; B: 0.0001 mg/L F^-. By method of IHC, the Cbfa1 protein in fibroblast was located in cytoplasm and the positive staining was only observed in the group of 0.0001 mg/L F^-).

F^- (Fig. 1). The Cbfa1 protein level in fibroblasts measured by ELISA was obviously increased at 48 h compared to the control group in the groups treated with 0.0001, 0.001, 0.1, 1.0 and 20.0 mg/L F^- to 2.35 ± 0.08, 2.28 ± 0.09, 2.32 ± 0.09, 2.25 ± 0.08, and 2.28 ± 0.09, respectively, and at 72 h in the group treated with 10 mg/L F^- (2.48 ± 0.22) (Fig. 2). In osteoblasts, the Cbfa1 protein level increased significantly at 4 h compared to the control group in the groups treated with 0.0001, 0.1, 1.0 and 20.0 mg/L F^- to 1.91 ± 0.06, 1.92 ± 0.12, 1.95 ± 0.05, and 1.89 ± 0.03, respectively, at 24 h in the group treated with 20 mg/L F^- (2.34 ± 0.09), at 48 h in the groups treated with 0.0001, 0.001, 0.1, 1.0, 10.0 and 20.0 mg/L F^- to 2.31 ± 0.14, 2.21 ± 0.21, 2.27 ± 0.08, 2.41 ± 0.00, 2.39 ± 0.19, and 2.39 ± 0.19, respectively, and at 72 h in the groups treated with 0.0001, 0.001, 0.1, 1.0, 10.0 and 20.0 mg/L F^- to 2.16 ± 0.22, 2.31 ± 0.09, 2.27 ± 0.16, 2.26 ± 0.09, 2.48 ± 0.27, and 2.23 ± 0.31, respectively (Fig. 3).

The expression of Cbfa1 mRNA in fibroblasts and osteoblasts treated with fluoride at 48 h

In fibroblasts, an increasing tendency in the expression of Cbfa1 mRNA was found in the fluoride groups, and the highest expression was found in the group treated with 10 mg/L F^- (1.29 ± 0.30, $p < 0.05$). In osteoblasts, the same tendency was found, and the highest expression was observed in the group treated with 0.0001 mg/L F^- ($p < 0.05$) (Fig. 4).

The OCN protein level in fibroblasts and osteoblasts treated with fluoride

Compared to the control group, the OCN protein level in fibroblasts was higher at 2 h in the groups treated with 0.0001, 0.1, 1, 10 and 20 mg/L F^- to 2.61 ± 0.22, 2.52 ± 0.18, 2.52 ± 0.18, 2.42 ± 0.29, and 2.42 ± 0.18, respectively and at 4 h in the groups treated with 0.001 and 0.1 mg/L F^- to 2.35 ± 0.08 and 2.35 ± 0.22, respectively. In contrast, the OCN protein level in fibroblasts was significantly lower than in the control at 48 h in the groups treated with 0.001 and 10 mg/L F^- to 2.06 ± 0.05 and 2.05 ± 0.12, respectively, and at 72 h in the groups treated with 0.0001, 0.1, and 1.0 mg/L F^- to 2.11 ± 0.10, 2.07 ± 0.11, and 2.05 ± 0.15, respectively. In osteoblasts, the OCN protein level increased obviously in all of the fluoride groups (Figs. 5 and 6).

The expression of OCN mRNA in fibroblasts and osteoblasts treated with fluoride at 48 h

In fibroblasts, the highest expression of OCN mRNA was found in the groups treated with 1 and 20 mg/L F^-, but these data had no

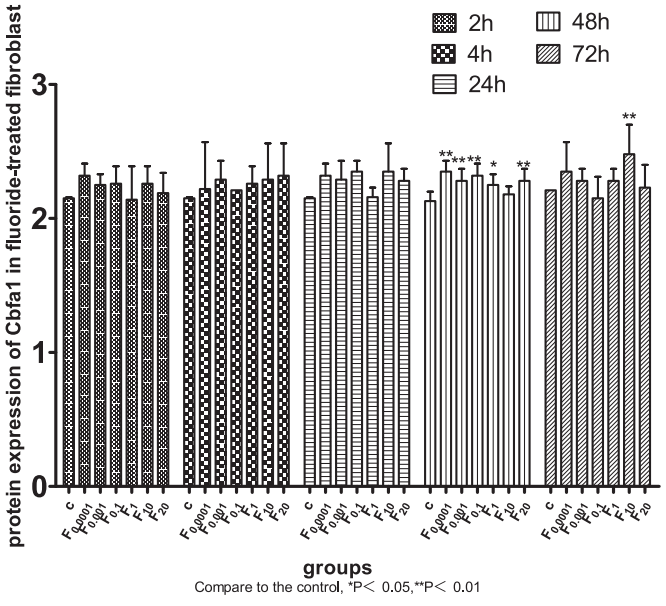

Fig. 2. The content of Cbfa1 protein in fluoride-treated fibroblast (The content of Cbfa1 protein in fibroblast measured by ELISA increased obviously in groups of 0.0001, 0.001, 0.1, 1.0 and 20.0 mg/L F^- at 48 h and group of 10 mg/L F^- at 72 h).

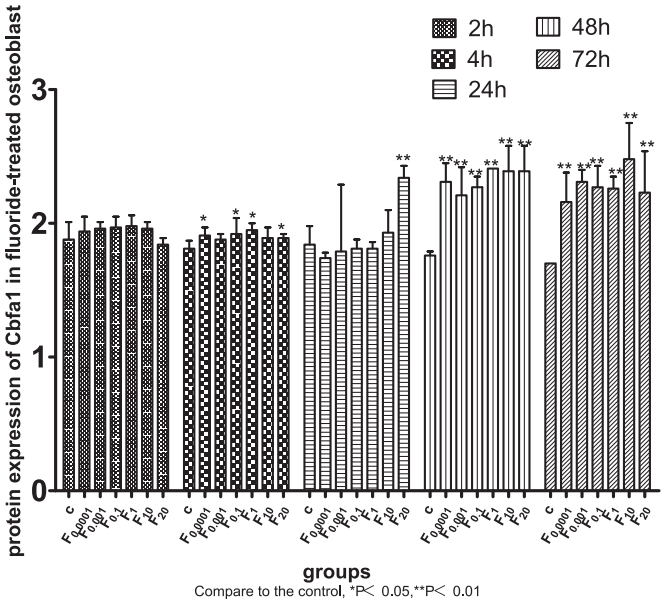

Fig. 3. The content of Cbfa1 protein in fluoride-treated osteoblast (Compared to the control group, the content of Cbfa1 protein increased significantly in groups of 0.0001, 0.1, 1.0 and 20.0 mg/L F^- at 4 h, 20 mg/L F^- at 24 h, 0.0001, 0.001, 0.1, 1.0, 10.0 and 20.0 mg/L F^- at 48 h, 0.0001, 0.001, 0.1, 1.0, 10.0 and 20.0 mg/L F^- at 72 h).

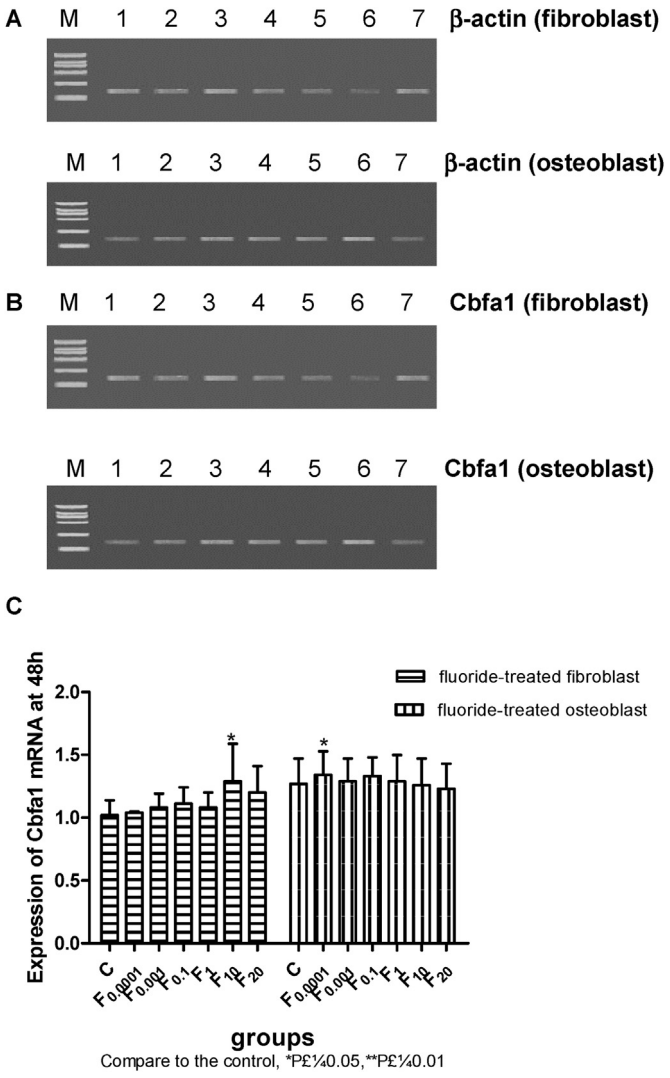

Fig. 4. Expression of Cbfa1 mRNA in fluoride-treated fibroblast and osteoblast at 48 h (A: bands of marker (β-actin) in fluoride-treated fibroblast and osteoblast at 48 h 1–7 were groups of 0.0001, 0.001, 0.1, 1, 10 and 20 mg/L F^- and control; M: marker, down to top: 100, 200, 300, 500, 900 and 1000 bp. B: bands of Cbfa1 mRNA in fluoride-treated fibroblast and osteoblast at 48 h, 1–7 were groups of 0, 0.0001, 0.001, 0.1, 1, 10 and 20 mg/L F^- and control; M: marker, down to top: DL1000 (100, 200, 300, 500, 900 and 1000 bp). C: expression of Cbfa1 mRNA in fluoride-treated fibroblast and osteoblast at 48 h. In fibroblast, the increasing tendency of express of Cbfa1 mRNA was found in fluoride groups and the highest expression was in group of 10 mg/L F^- ($p < 0.05$); In osteoblast, the same tendency was showed and the highest expression was observed in group of 0.0001 mg/L F^- ($p < 0.05$)).

statistical significance. In osteoblasts, the expression of OCN mRNA increased slightly in all fluoride groups except for the 20 mg/L F^- group (Fig. 7).

Discussion

Two osteoblast-specific cis-acting elements (OSEs) were identified in the promoter of the OCN gene: OSE1 and OSE2 [8,15]. This led to the identification of Cbfa1 as the first osteoblast-specific transcription factor. During embryonic development, Cbfa1 expression precedes osteoblast differentiation and is restricted to the transition of mesenchymal cells to chondrocytes or osteoblasts [16–19]. Subsequently, as the earliest and most specific marker of osteogenesis, Cbfa1 expression is a particular marker of osteoblasts [20] and has a lower level of expression in hypertrophic chondrocytes. In cell culture, Cbfa1 acts as an activator of transcription and can

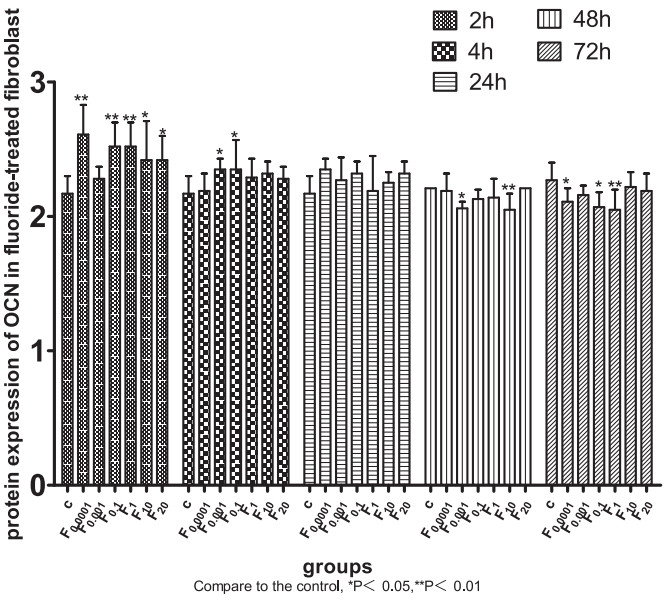

Fig. 5. The content of OCN protein in fluoride-treated fibroblast (Compared to the control group, the content of OCN protein in fibroblast was higher in groups of 0.0001, 0.1, 1, 10 and 20 mg/L F^- at 2 h, 0.001, 0.1 mg/L F^- at 4 h, while that was lower significantly in group of 0.001, 10 mg/L F^- at 48 h, 0.0001, 0.1 and 1.0 mg/L F^- at 72 h).

induce osteoblast-specific gene expression in fibroblasts and even myoblasts. In addition to its critical role during osteoblast differentiation, Cbfa1 controls bone formation by differentiated osteoblasts. As noted above, Cbfa1 regulates the expression of OCN, a gene expressed only in terminally differentiated osteoblasts. Cbfa1 binding sites are also present in the regulatory sequences of most genes required for the elaboration of a bone extracellular matrix. Cbfa1 is mainly expressed in osteoblasts and is the most specific and important transcription factor that has been discovered thus far for osteoblast differentiation and bone formation. Cbfa1 can regulate the synthesis of the extracellular matrix, the precipitation of the bone matrix and the expression of the OCN gene. OCN, a major non-collagenous matrix protein secreted by osteoblasts [21]

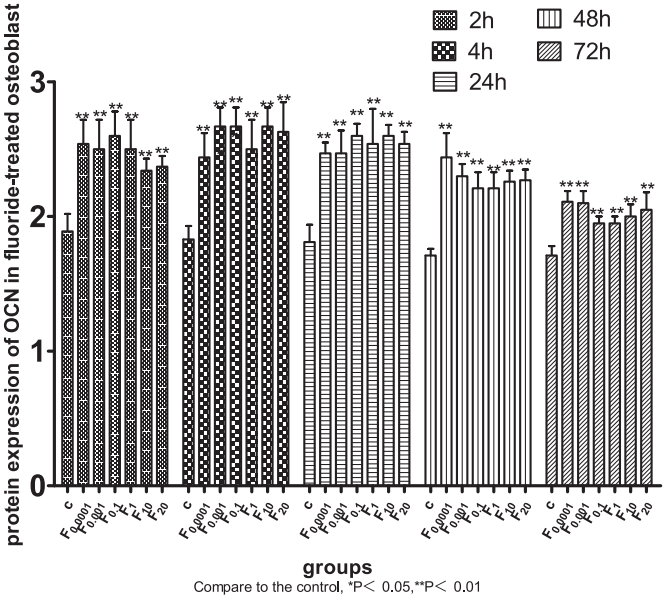

Fig. 6. The content of OCN protein in fluoride-treated osteoblast (In osteoblast, the content of OCN protein increased obviously in all of fluoride groups ($p < 0.05$)).

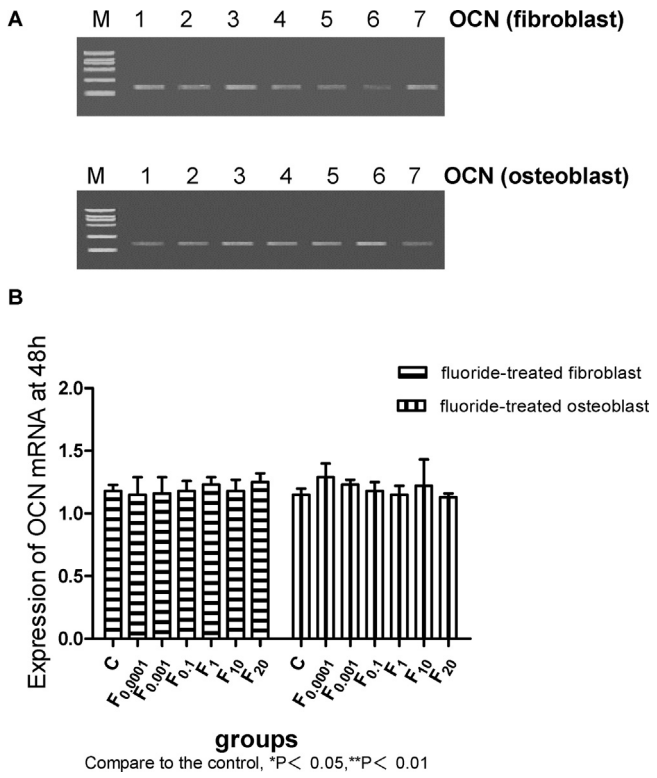

Fig. 7. The expression of OCN mRNA in fibroblast and osteoblast exposed to fluoride at 48 h (A: bands of OCN mRNA in fluoride-treated fibroblast and osteoblast at 48 h, 1–7 were groups of 0, 0.0001, 0.001, 0.1, 1, 10 and 20 mg/L F^- and control; M: marker, down to top: DL2000 (100, 250, 500, 750, 1000, 1600 and 2000 bp). B: expression of OCN mRNA in fluoride-treated fibroblast and osteoblast at 48 h. In fibroblast, the higher expression of OCN mRNA was found in groups of 1, 20 mg/L F^- but having no statistical significance. In osteoblast, the expression of OCN mRNA increased slightly in all fluoride groups excepting for the group of 20 mg/L F^-).

and odontoblasts, was discovered in the mid-seventies by Price and coworkers [22] and was found to be in tight association with the calcium phosphate mineral phase of bone, dentin, and cementum. FBs are considered terminally differentiated cells, but the dedifferentiation or cell phenotype transformation of FBs can occur as the result of a repressed gene being reactivated or an abnormal gene being activated under non-physiological conditions. We deduce that FBs can transform into OBs because both FBs and OBs have the same origin, they have a high level of cell phenotype overlap and they differentiate in a similar manner. OBs from patients with osteoporosis have been shown to present a fibroblast-like appearance, indicated by the partial loss of expression of ALP, OCN and CO I and the expression of non-specific collagen III. Many reports show that FBs exhibit an OB phenotype after activating the Cbfa1 gene induced by bFGF, BMP_2, TNF and so on.

Both osteoblasts and fibroblasts are of mesenchymal origin. The only morphological feature specific to osteoblasts is located outside the cell in the form of a mineralized extracellular matrix. All the genes expressed in fibroblasts are also expressed in osteoblasts except for two osteoblast-specific transcripts that have been identified: Cbfa1 and OCN. The osteoblast can be viewed as a sophisticated fibroblast [23]. Human skin fibroblasts can be induced to a pro-osteoblast state by expressing Cbfa1 in combination with TNF-α and BMP-2 [24]; the expression of ALP and OCN has been observed in rabbit skin fibroblasts after being transfected with the Cbfa1 gene [25]. In osteoporosis patients, osteoblasts were found to be similar to fibroblasts morphologically and to synthesize ALP, OCN and COL I at low levels. Furthermore, fibroblasts from the joint in osteoporosis patients were capable of transforming into osteoblasts under the effect of BMP-2 and TGF-β.

It is well known that fluoride is a factor that seems to particularly alter the activity of osteoblasts, both systemically and locally or through their presence in apatite crystals. Fluoride also seems to change the amount of bone mass in rats in a dose-dependent manner, and has been used in protocols for the treatment of osteoporosis. Some research has provided evidence that fluoride acts directly on osteoblasts to impair early extracellular matrix biosynthesis and is associated with the activity and maturation stage of intrinsic osteoblasts [26].

We propose that the expression of Cbfa1 and OCN in fibroblasts can be induced by fluoride resulting in the stimulation of the osteogenic function of fibroblasts. There is no united opinion about whether other cells except for osteoblasts can express “Cbfa1” and “OCN” or not. Most of academics think that there is no expression of “Cbfa1” and “OCN” in FBs under physical conditions, but some researcher argues that there has a weak expression in skin FB [27]. By using ELISA and IHC methods, the Cbfa1 and OCN protein levels in osteoblasts and fibroblasts and in cell culture fluid were measured. A similar increasing tendency in the level of Cbfa1 protein was found in fibroblasts and osteoblasts. However, in osteoblasts, the increase was much greater and lasted much longer than in fibroblasts. Fluoride also up-regulated the expression of the OCN protein in fibroblasts and osteoblasts, and this effect was much stronger in osteoblasts.

The expression levels of Cbfa1 and OCN mRNA were detected using RT-PCR and were found to be increased by fluoride treatment.

When Cbfa1 is expressed in non-skeletal cells, the cells assume many of the characteristics of osteoblasts. For example, the secretion of OCN by ankylosing spondylitis (AS) fibroblasts is higher than that of normal fibroblasts. The facts discussed above show that AS fibroblasts have the same biological characteristics as osteoblasts. A higher expression level of OCN indicates the enhancement of osteogenesis. Our results indicated that fluoride up-regulated the expression of Cbfa1 and OCN mRNA and protein in fibroblasts as well as osteoblasts. These results show that skeletal fluorosis is mainly characterized by a high bone turnover state due to active osteoblasts. On the other hand, these results also presented the following clues: (1) the expression of Cbfa1 and OCN exist even in terminally differentiated cells, such as fibroblasts, but in a weak degree; (2) fluoride obviously enhances the levels of Cbfa1 and OCN.

Abnormal intramembranous ossification and endochondral ossification can be induced by both the decreased and increased expression of Cbfa1. Fibrodysplasia ossificans progressive (FOP), a rare genetic disease, is characterized by sporadic ectopic ossification in muscle. The expression of Runx2/Cbfa1 in FOP cells was observed in the early stages of the disease. Extraperiosteal soft tissue is composed of dense connective tissue that has no osteogenic capability in the physiological environment. Our results suggest that fluoride enhances the mRNA and protein levels of Cbfa1 and OCN.

This paper is the first to report that fluoride can induce the osteogenic phenotype in FBs. These results coincided with our hypothesis. The studies discussed above demonstrated the critical function of Cbfa1 in the pathogenesis of extraperiosteal ossification. Determining the causal relationships between fluoride, FBs and extraperiosteal ossification will help elucidate the mechanism of the promotion of osteogenesis of FBs by fluoride. Future work in this area will explain the pathogenesis of calcification and osteogenesis in extraperiosteal tissues and help establish the scientific policy for protecting disabled skeletal fluorosis; it will also give aid in the study of other similar diseases.

Conflict of interest

All authors state that they have no conflicts of interest.

Authors' contributions

Study design: Ling Jing, Guangsheng Li, Hui Xu; Study conduct: Xiaoqin Duan, Hui Xu, Ying Wang, and Huan Wang; Data collection and analysis: Xiaoqin Duan, Ying Wang, Huan Wang; Data interpretation: Guangsheng Li, Ling Jing; Drafting manuscript: Xiaoqin Duan; Revising manuscript content: Guangsheng Li, Ling Jing; Approving final manuscript: Xiaoqin Duan, Guangsheng Li, Ling Jing.

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